-7-737 (-)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 91/08292
C12N 15/29, 15/82, A01H 4/00	A1	(43) International Publication Date: 13 June 1991 (13.06.91)

(21) International Application Number: PCT/FI90/00284

(22) International Filing Date: 23 November 1990 (23.11.90)

(30) Priority data:
895614 23 November 1989 (23.11.89) FI

(71) Applicant *(for all designated States except US)*: KEMIRA OY [FI/FI]; PL 44, SF-02271 Espoo (FI).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : FRANCK, Marianne [FI/FI]; Pietarinkatu 2 C 27, SF-00140 Helsinki (FI). KURKELA, Sirpa [FI/FI]; Pengerkatu 21 A 39, SF-00500 Helsinki (FI).

(74) Agent: BERGGREN OY AB; P.O. Box 16, SF-00101 Helsinki (FI).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), SU, US.

Published
With international search report.
In English translation (filed in Finnish).

(54) Title: DNA MOLECULES IMPROVING COLD-RESISTANCE

(57) Abstract

The present invention relates to a novel gene which was isolated from the plant *Arabidopsis thaliana L.* More particularly, the present invention relates to a DNA molecule which comprises a structural gene which encodes a cold hardening protein or a protein which has similar biological properties and is substantially homologous with the said protein, and the regulating region of the structural gene.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo			SN	Senegal
CH	Switzerland	KR	Republic of Korea	SU	Soviet Union
CI	Côte d'Ivoire	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

DNA molecules improving cold-resistance

The present invention relates to a novel cryoprotective gene which has been isolated from the plant Arabidopsis thaliana L.

In spite of the large amount of biochemical and physiological data available on the cold resistance of plants, what really happens to a plant in cold is not yet fully understood. For example, it is not known why certain plants tolerate freezing and others do not, or what the primary reason for cold damage is. An ability to improve the cold resistance of plants would provide considerable advantages for agriculture in cold regions of the globe.

The cold resistance of a plant is not a constant state but develops when the plant is exposed to low non-freezing temperatures (= acclimation). Although a number of cold acclimation specific changes have been observed in mRNA and in polypeptide profiles, very little is known about the action of these cold inducible proteins or about the mechanisms regulating these changes. So far, acclimation specific genes have not been isolated.

The object of the present invention is thus to isolate a cold hardening gene which can be transferred by genetic engineering methods into some crop plant and be induced to produce a protein which will enhance the resistance of the plant to cold.

In order to obtain additional information regarding the genetic and molecular bases of the cold hardening process of plants, cold acclimation specific genes from Arabidopsis thaliana were investigated in connection with the present invention. It was observed that Arabidopsis is cold resistant, and that this cold resistance is associated with a number of changes at the level of gene expression.

The present invention relates to a novel gene which was isolated from the plant Arabidopsis thaliana L. More particularly, the present invention relates to a DNA molecule comprising a structural gene which codes for a cold hardening protein or for a protein which has similar biological properties and is substantially homologous to the said protein, and its regulating region which responds to a drop in temperature.

It was observed that the gene kin1 was induced in six hours at +4 °C and acted as long as the plant was kept in cold and became inactive in 12 h when the plant was transferred back to the control temperature. The gene is also inducible by water stress and salt stress and by abscisic acid (ABA). ABA is a plant hormone which functions as a mediator in various plant stress situations, and it has been shown also to contribute to the cold acclimation of plants. ABA (50 µM) has been observed to raise the induction level to as high a level as does cold.

A genomic library was constructed in EMBL3, and cold inducible genes were identified by differential hybridization. One gene, the one which was induced most clearly, was selected for further characterization. A genomic clone was used as a probe for finding the corresponding cDNA clone in the enriched library which had been constructed in the plasmid pUEX1. Both clones were sequenced, the transcription initiation site was determined by the primer extension method, and the polyadenylation site from the cDNA sequences. The nucleotide sequence of the genomic clone is shown in Figure 1. The assumed regulating region of the gene is underlined, starting from the base pair 720 and ending with the base pair 2132. After this base pair there begins the actual structural gene of the DNA molecule. The cDNA sequence and the corresponding amino acid sequence are shown underlined in Figure 2.

The longest open reading frame (ORF) of the cDNA sequence,

starting from the first ATG downstream from the transcription initiation site, encodes a protein of 65 amino acids having a predicted molecular mass of 6478 and an isoelectric point of 7.2. The amino acid sequence of the protein is shown in Figure 3. Hybrid selection experiments showed that the kin1 clone hybridizes to mRNA(s) which encode a polypeptide of similar size. The protein is rather hydrophilic, and it has a high amount of α-helical structure. Its amino acid composition is rather unusual: 22.4 % Ala, 13.4 % Gly and 13.4 % Ser. The gene contains the 5' and 3' untranslatable regions of the 62 and 117 nucleotides, respectively, and two introns.

The invention is described below in greater detail.

1) Isolation of a cold-inducible gene from the genomic library of the plant *Arabidopsis thaliana*

For the genomic library, the total DNA of the plant *Arabidopsis thaliana* was fragmented partially by means of SAU3a restriction enzyme, and fragments of 15-20 kb were isolated from agarose gel. The fragments were ligated to BamH1 enzyme restricted arms of the vector λEMBL3, and the ligation mixture was packed *in vitro* inside the λ particles by a method known *per se* (Maniatis et al., Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982). The *E. coli* host was infected with λ particles, and the recombinant phases which contained the cold-inducible gene were identified by so-called differential hybridization (on the dish, the phages formed plaques from which the DNA was transferred, by the method of Maniatis et al., to the filters used in the hybridization). The probes used in the differential hybridization consisted of cDNA made from RNA isolated from the control pl ts. For further experiments, a recombinant was selected which yielded in the hybridization a signal with cold cDNA but not with control cDNA. The fragment containing the gene was transferred from the λ vector to the pUC18 vector, and the more precise location of the gene in the fragment was determined by

differential hybridization. The 4369 kb fragment was sequenced.

2) Preparation of cDNA

cDNA corresponding to the genomic clone was captured from a cDNA library prepared from the cold RNA. The synthesis of cDNA from messenger RNA and its cloning into the vector pUEX1 were carried out using Amersham kits (RPN 1256 and RPN 1282). The probe used in the hybridization was a genomic clone. The cDNA was sequenced (Figure 2).

3) Southern blot analysis of DNA

Southern blotting was carried out by the method of Maniatis et al. (1982). The results of the genomic Southern blot test and the sequence analysis of cross-hybridizing cDNA clones shows that the gene is present in two copies in the genome of Arabidopsis.

4) Northern blot analysis of RNA

The cold-inducibility of the gene kin1 was demonstrated by Northern blotting. The northern blot analysis was carried out by the method of Maniatis et al. (1982). Northern blotting was used for analyzing the steady state levels of kin1 mRNA during acclimation. Total RNA was isolated from the control plants and from the plants kept at a low temperature, by using the method described in Jones et al., High level expression of introduced chimeric genes in regenerated transformed plants, EMBO J. 4, pp. 2411-2418. The total RNA was analyzed carefully by the method of Maniatis et al., 1982. The results are shown in Figure 4. The figure shows that at low temperatures the amount of RNA corresponding to the kin1 gene is approx. 15-20 times the amount that is present at the control temperature, +22 °C, which means that the gene is cold inducible.

kin1 mRNA was detectable 6 hours after the transfer to a low temperature, and the level remained high throughout the 7-day acclimation phase. It was observed that the induction was cold

specific, i.e. when the plants were transferred back to the control temperature, the amount of kin1 mRNA dropped in 12 hours.

5) Investigation of the induction properties of the cold gene

a) Abscisic acid (ABA)

Since the adding of exogenous abscisic acid (ABA) induces cold resistance in a number of plant species which are cold resistant, and an increase of endogenous ABA levels has been observed during this time, an investigation was made as to whether the kin1 gene would respond to ABA. The experiment was carried out as in point 4 above, by Northern blot analysis. The probe used was kin1 cDNA. Figure 5 shows the results of the effect of ABA (10 mM and 100 mM) on the expression of the kin1 gene. It was observed that both the spraying of *Arabidopsis* with 100 µM ABA and watering it with 10 µM ABA caused kin1 induction. This was the first time that direct molecular evidence of the role of ABA in cold acclimation was observed.

b) Water stress

An investigation was made as to whether the gene kin1 is inducible by water stress. The lowering of the cell water potential is common to this stress. It has been demonstrated that the damage caused to spinach leaves by wilting is similar to the dehydration caused by freezing. It has also been thought that tolerance of freezing is due to avoidance or tolerance of the dehydration caused by freezing. The dehydration is caused by ice formed in the extracellular space "sucking" the water out from inside the cell. It has also been noted that cold resistance can be induced in plants by mere desiccation stress, without exposure to low temperatures. When plants were exposed to desiccation of different degrees, it was observed that wilting really did induce the kin1 gene.

c) Salt stress

A high intercellular salt concentration results in water leav-

ing the cell, with dehydration resulting from it. An investigation was made whether the kin1 gene was inducible by salt stress (300 mM NaCl). The result of a Northern blot analysis shows that the kin1 gene is inducible also by salt stress (Figure 6).

6) Making of DNA constructs which are capable of action in plants, and their transfer into plants

The activity of the kin1 gene was investigated in cold-sensitive, non-acclimating tobacco plants (*Nicotiana tabacum* SR1) and *Arabidopsis*, by using two different DNA constructs. The first construct (p35S-sense-kin1) contains the kin1 gene with the cDNA in the correct orientation (so-called sense), regulated by a strong, continuously acting cauliflower mosaic virus 35 S transcript regulating region (p35S; Fromm M. et al., 1985. Proc. Natl. Acad. Sci. USA, 82:5824-5828). In this manner the activity of the kin1 gene is in all parts of the plant independent of the temperature and the gene is also active in other plant species. In the other construct (p35S-antisense-kin1), the cDNA of the kin1 gene is in the wrong orientation (so-called antisense), regulated by p35S. The activity of the kin1 gene can be eliminated by transferring this construct into *Arabidopsis*, from which kin1 has been isolated. Thus it can be determined whether or not the kin1 gene is indispensable in the acclimation and cold resistance of *Arabidopsis*.

a) p35S-sense-kin1

The BamHI-BclI fragment of the pUEX1-cDNA clone was ligated to BamHI-digested plasmid pHTT203. The result was the plasmid pSKH100 (Figure 7), in which the kin1 cDNA was in the correct orientation under p35S regulation and which had the known neomycin phosphotransferase 2 nopaline synthase gene (pnos-npt2), used as the selectable marker, for fusion to the regulating region, and the known marginal areas of T-DNA which are needed for the transfer of DNA to a plant.

b) p35S-antisense-kin1

The NcoI fragment of t .EX1-cDNA clone was ligated to BamHI digested plasmid pHTT2... The result was the plasmid pSKH107 (Figure 8), in which kin1 cDNA was in the wrong orientation under p35S regulation and which had the pnos-npt2 gene used as the selectable marker and the known marginal regions of T-DNA.

The p35S-sense-kin1 construct was transferred into tobacco plants and the p35S-antisense-kin1 construct was transferred to Arabidopsis plants by using a known Agrobacterium-mediated transfer method (Hernalsteens J.P. et al., 1980, Nature 287: 654-656; Valvetens D. et al., 1988, Proc. Natl. Acad. Sci. USA, 85:5536-5540).

7) Investigation of the act.on of the cold gene

a) In tobacco

Tobacco does not have the kin1 gene nat...ally. In transgenic plants into which the construct p35S-s -kin1 has been transferred it was observed that the transfe..ed recombinant gene is present and is detectable by the Northern blot analysis (Figure 9). In cold resistance tests, ion bleedi: of tobacco leaf fragments is measured as a function of the temperature, by a known method (Sukumaran & Weiser, 1972, Hortscience 7:467-468). The plants are regarded as dead when a 50 % ion bleeding occurs. It was observed that the transgenic tobacco had a cold resistance approximately 1.2 degrees greater than had the control plant (Figure 10). This indicates that, when transferred into a cold-sensitive plant, the kin1 gene improves the cold resistance of the plant concerned.

b) In Arabi..psis

p35S-antisense-kin1 constructs were investigated in Arabidopsis. The cold resistance test on the transgenic plants (Figure 11) shows that the acclimation ability of p35S-antisense-kin1 plants is significantly lowered. These observations demonstrate that the kin1 gene affects the cold resistance of Arabidopsis

and is indispensable for it.

8) Investigation of the regulating region
A genomic clone fragment Hind3-BsmI which contained regulation region was ligated to the glucuronidase gene (gusA; Jefferson R.A. et al., 1987, J. EMBO 6:3901-3907), and this fusion was transferred into a known vector which contained the selectable marker puos-npt2 and the marginal regions of T-DNA. The thus obtained construct pkin1-gusA in the plasmid pMEG3 (Figure 12) was transferred into tobacco plants by Agrobacter mediation.

The presence of the construct in a tobacco plant, and its activity, were investigated by determining the gus activity by a known method. Table 1 shows that pkin1 acts as the regulating region in tobacco, and its activity is higher at a low temperature.

Table 1

	A 415nm	
	+22 °C	+4 °C
Control	0.01	0.01
pkin1-gusA	0.281	0.830

Claims

1. A DNA molecule, **characterized** in that it comprises a structural gene which encodes a cold hardening protein, and the regulating region of the gene.

2. A DNA molecule according to Claim 1, **characterized** in that the structural gene encodes a cold hardening protein isolated from the plant Arabidopsis thaliana L., or a protein which has similar properties and is substantially homologous with the said protein.

3. A DNA molecule according to Claim 1 or 2, **characterized** in that it is derived from Arabidopsis thaliana L.

4. A DNA molecule according to any of the above claims, **characterized** in that it has the base sequence shown in Figure 1, or part thereof.

5. A DNA molecule according to any of the above claims, **characterized** in that its structural gene has a base sequence which corresponds to the bases 2136-3100, or a part thereof, in Figure 1.

6. A DNA molecule according to Claim 1, 3 or 4, **characterized** in that its regulating region has a base sequence which corresponds to the bases 720-2132, or a part thereof, in Figure 1.

7. A DNA molecule according to any of the above claims, **characterized** in that its structural gene encodes a protein the amino acid sequence of which is depicted in Figure 3, or a protein of equivalent biological activity, having substantially the amino acid sequence depicted in Figure 3.

8. A DNA molecule according to Claim 5 or 6, **characterized** in that additional bases are ligated to the 5' and 3' ends of

the said base sequence.

9. A cDNA molecule, **characterized** in that it has the base sequence depicted underlined in Figure 2.

10. A crop plant or decorative plant, **characterized** in that a DNA molecule according to any of Claims 1-8 has been incorporated into it by genetic engineering methods.

11. A crop plant or decorative plant, **characterized** in that a cDNA molecule according to Claim 9 has been incorporated into it by genetic engineering methods.

```
  1 CCTGCACCTC GACTCTAGAG GATCCCCGGG TACCGAGCTC GAATTCGGAT    50

 51 GATCCAAATG TTGAATATAA TGGATCAAGT ATCTATTATT ATGATCATTT   100

101 TCATGATTTT AGTGTATTAA GCTAATTGCT TTGCATTTGA CTTTAAATCT   150

151 CATGACACCT TTGGTTTTTC ACATAGTTGA AATCTAGCTT AACCTCATAG   200

201 TTAACAAGGT CATAATATTT CGCCGAGGCA TAATAATCGA AAGAGTCAAA   250

251 GAAAGCGAGA GACGCACGAT AGAAAAAAAA AACTAGATAT ATTAAAAAAA   300

301 TTTTTTTTTT TTTTAGATAT ATTAATTTGA CTAAACGGCT ACGCTTTGTG   350

351 CCCAGTCTTC GTAGAGAATC GCTACGTTTT TATTGTAACC GAAACAAGAC   400

401 AAGAAACGTG TTTTCGATTG TGTTTGTGTA TGCCGATAAA CTATTATATT   450

451 TTTGTAATTT GATTTAGTTC AAGAAATTGT TGTGGCCTAT CGGCTATCAA   500

501 CATGCTTTTT GTAAGAGAGA ACTTGTAATT TCTGTCTATG ATTTCCTTCG   550

551 TTTTCTTTTT CTCTCTCAAA GTCATTTATT TAAGAACAA CATTACGTGT    600

601 GTATAATTGT ATAATTTTTT CTATTTCTTA CTATACTTAG AGAATTACAT   650

651 GTTATAACAA ATGATCTTTG ATCAAAAGAA AACAACAAC AAATGATCAA    700

701 GAATTGCTTT CTTTTTTTTA TATGTTGCAA AATGAATAGA CGAGCCAAAC   750

751 TTATACTCAA ATATTGTTTT ATCTATTTTT AAGAAATATT CTTTATGCGA   800

801 GAGAGCAAAC TCCCCAAACG ATGTTAAATG GATCACAACA CATAAGTCGA   850

851 TCCGAATAGT TGAAGTTTTC TAAAAAGGCA GTCCCTATAA TTGTATTAAA   900

901 CACACTAAAT CCTCCACACA CACAAAAGGA AATCACGTAA ACGAGACCTT   950

951 TACATGAAAC AATCAAACCA GTTTGATTTT ATCCGATTTT GCTAATGAGA  1000
```

FIG. 1

1001 TGAGTTCTGT ATATTGTTAT AATGGATATA GGAGAACTAA CTAAAATAAA 1050

1051 AAACGCAAAG AAAACTTTAT ACAGTACATA TTCTTATGGT CTTACCAATT 1100

1101 ATCAACGATG TGTTGGTTAT GTACACAAGG TAAAATACAT CGATTCATAT 1150

1151 TATGCATAAT ATAATGGGAA AATAAAGAAT AAAACTACAT TTTGGTAACT 1200

1201 TGAATTCAAC CATGAACTGT TTGGATTGGC AAACATAAAC TCAAATAAAA 1250

1251 TATCTAGGTA TAATTGTGGT TCATACAAGA ATTACTTCAT ACTGTTGGGC 1300

1301 CAAAGGGTAC GTATCCTTCC CCGCACCTCC AAACCATGGG CTTACTACTG 1350

1351 ATCCGACATC AAAACCGTGT TAGTTGCAAC CAACGAATGA TAAGTCAATA 1400

1401 AGATTCAACT TGTCAACAAA TATACAGCTT ATATGACATG TCTGGCTCCA 1450

1451 AACTGAATTT TAGTAGAAAG TTACTAATTC ATAAAATTAA TTTATATACA 1500

1501 ATTTTTCAAT TTTTATTTTA TAAATTAAAG AAAAAAACAT GAAAAATACG 1550

1551 GGAGGTTCGG CAAACACAAC ATTTAACTTG CCAAACGTAT CATCTAACTT 1600

1601 TCCCACCTTA TACAAGGAAC CATTTTTTCA ATAATAAAGT TTTTTTTTTT 1650

1651 TTTTGTCTTC GCAAATAAGA GCACGAAATG TTTGCCAAAC GCATATGCAA 1700

1701 CAAACCCACG TTACATAATT CTGTTTACAG CCATAGAGCA AGCTATATTG 1750

1751 TTAAAGACCT AAAAAAAATC TTTACTATAA CATATAGAGG CTTCGAGATA 1800

1801 TTTCGAAAGA CTCAACTTAT ATATAAATAA ACTCAAAAAG AAAACACGGA 1850

1851 GGCGAGAGGA TCATACTCTC ACACAGAAAG AGTCACATTA TTATATCCTC 1900

1901 TAAAAAACCA AACTAAAACG ACACGTGAAG TCTTGATCAG CCGATAAATA 1950

1951 GCTACCGACA TAAGGCAAAA CTGATCGTAC CATCAAATGT AATCCACGTG 2000

2001 GTTTTAGATT ACTCGTGGCA CCACACTCCC TTTAGCCTAT AAATATAAAC 2050

2051 CATTAAGCCC ACATCTCTTC TCATCATCAC TAACCAAAAC ACACTTCAAA 2100

2101 AACGATTTTA CAAGAAAAAA ATATCTGAAA AAATGTCAGA GACCAACAAG 2150

2151 AATGCCTTCC AAGCCGGTCA GACCGCTGGC AAAGCTGAGG TACTACTCTT 2200

2201 TCTCTCTTTG ACAGAACTCT TAAACTGGAA AAATTGTTGA AGCTATAACT 2250

2251 CTTTGAAAAC AGTTGAAACT TGATCATTAC TAGAAATTTC AGTTACTTGT 2300

2301 TTAATTTAGT TTGTCGTAAT TATGTAATTG ATGATTTTAT GGTTACAATG 2350

2351 GTTGTCATGT AGGAGAAGAG CAATGTTCTG CTGGACAAGG CCAAGGATGC 2400

2401 TGCAGCTGGT GCTGGAGCTG GAGCACAACA GGTAAACAAT CCATACACAG 2450

2451 ACACATAACA TATAATATGT AACGAAATAA ACGTCTTTGT AAGCTTACAT 2500

2501 GTACGCAGAT TTCTGATATG GTTATGTATA TGTTATAGGC GGGAAAGAGT 2550

2551 GTATCGGATG CGGCAGCGGG AGGTGTTAAC TTCGTGAAGG ACAAGACCGG 2600

2601 CCTGAACAAG TAGAGATTCG GGTCAAATTT GGGAGTTATA ATTTCCCTTT 2650

2651 TCTAATTAAC TGTTGGGATT TTCAAATAAA CGATCTTTGA TCAAGAATTG 2700

2701 CATTATATAT ATATATATAA AAATATATTG CAAAATTATT AGACGAGCCA 2750

2751 AACTTATATT CAAATAATGT TTTATCTATT TTAAAAAATA TTCTTTATGC 2800

2801 GAAAGATCAA ACTCCCCAAA CGATGTTAAA TGGATCACGA TACATAAGTC 2850

2851 GATCCGAATT GTTGAAGTTT TCTAAAAATG CAGTCCTTAT AATTGTATTA 2900

2901 AACACACTAA ATCTTCCAAA CACACAGAAG GAAATCACGT AAACGAGACC 2950

2951 TTTACATGAA ACTATCAAAC CAGTTTGAGT TTATCCGATT TTGCTAATGA 3000

3001 GACGAGTTCT ATATATTGTT ATAATGGATA TAGGAGAACT AACTAAAAAA 3050

3051 AAACGCAAAG AAAACTTTAT ACATATTCTT ATGGTCTTAC CAATAATCAA 3100

```
2001 GTTTTAGATT ACTCGTGGCA CCACACTCCC TTTAGCCTAT AAATATAAAC  2050

2051 CATTAAGCCC ACATCTCTTC TCATCATCAC TAACCAAAAC ACACTTCAAA  2100

2101 AACGATTTTA CAAGAAATAA ATATCTGAAA AAATGTCAGA GACCAACAAG  2150
                                      MetSerGl uThrAsnLys

2151 AATGCCTTCC AAGCCGGTCA GACCGCTGGC AAAGCTGAGG TACTACTCTT  2200
     AsnAlaPheG lnAlaGlyGl nThrAlaGly LysAlaGl

2201 TCTCTCTTTG ACAGAACTCT TAAACTGGAA AAATTGTTGA AGCTATAACT  2250

2251 CTTTGAAAAC AGTTGAAACT TGATCATTAC TAGAAATTTC AGTTACTTGT  2300

2301 TTAATTTAGT TTGTCGTAAT TATGTAATTG ATGATTTTAT GGTTACAATG  2350

2351 GTTGTCATGT AGGAGAAGAG CAATGTTCTG CTGGACAAGG CCAAGGATGC  2400
                uGluLysSe rAsnValLeu LeuAspLysA laLysAspAl

2401 TGCAGCTGGT GCTGGAGCTG GAGCACAACA GGTAAACAAT CCATACACAG  2450
     aAlaAlaGly AlaGlyAlaG lyAlaGlnGl

2451 ACACATAACA TATAATATGT AACGAAATAA ACGTCTTTGT AAGCTTACAT  2500

2501 GTACGCAGAT TTCTGATATG GTTATGTATA TGTTATAGGC GGGAAAGAGT  2550
                                             nAl aGlyLysSer

2551 GTATCGGATG CGGCAGCGGG AGGTGTTAAC TTCGTGAAGG ACAAGACCGG  2600
     ValSerAspA laAlaAlaGl yGlyValAsn PheValLysA spLysThrGl

2601 CCTGAACAAG TAGAGATTCG GGTCAAATTT GGGAGTTATA ATTTCCCTTT  2650
     yLeuAsnLys TER

2651 TCTAATTAAC TGTTGGGATT TTCAAATAAA CGATCTTTGA TCAAGAATTG  2700

2701 CATTATATAT ATATAAATAT ATTGCAAAATT ATTAGACGA GCCAAACTTA  2750

2751 TATTCAAATA ATGTTTTATC TATTTTAAAA ATATTCTTT ATGCGAAAGA  2800
```

FIG. 2

Met Ser Glu Thr Asn Lys Asn Ala Phe Gln Ala Gly Gln Thr Ala Gly Lys 17
Ala Glu Glu Lys Ser Asn Val Leu Leu Asp Lys Ala Lys Asp Ala Ala Ala 34
Gly Ala Gly Ala Gly Ala Gln Gln Ala Gly Lys Ser Val Ser Asp Ala Ala 51
Ala Gly Gly Val Asn Phe Val Lys Asp Lys Thr Gly Leu Asn Lys TER

FIG. 3

A B C

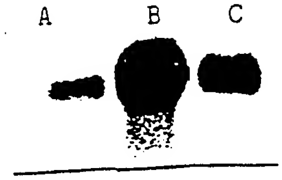
A = +22°C
B = +4°C, 1 day
C = +4°C, 2 day

FIG. 4

A = +22°C
B = +4°C, 1 day
C = 10 mM ABA, "
D = 100 mM ABA, "

FIG. 5

A B C



A= +22°C
B= +4°C, 1 day
C= +22°C, 300 mM NaCl, 1 day

FIG. 6

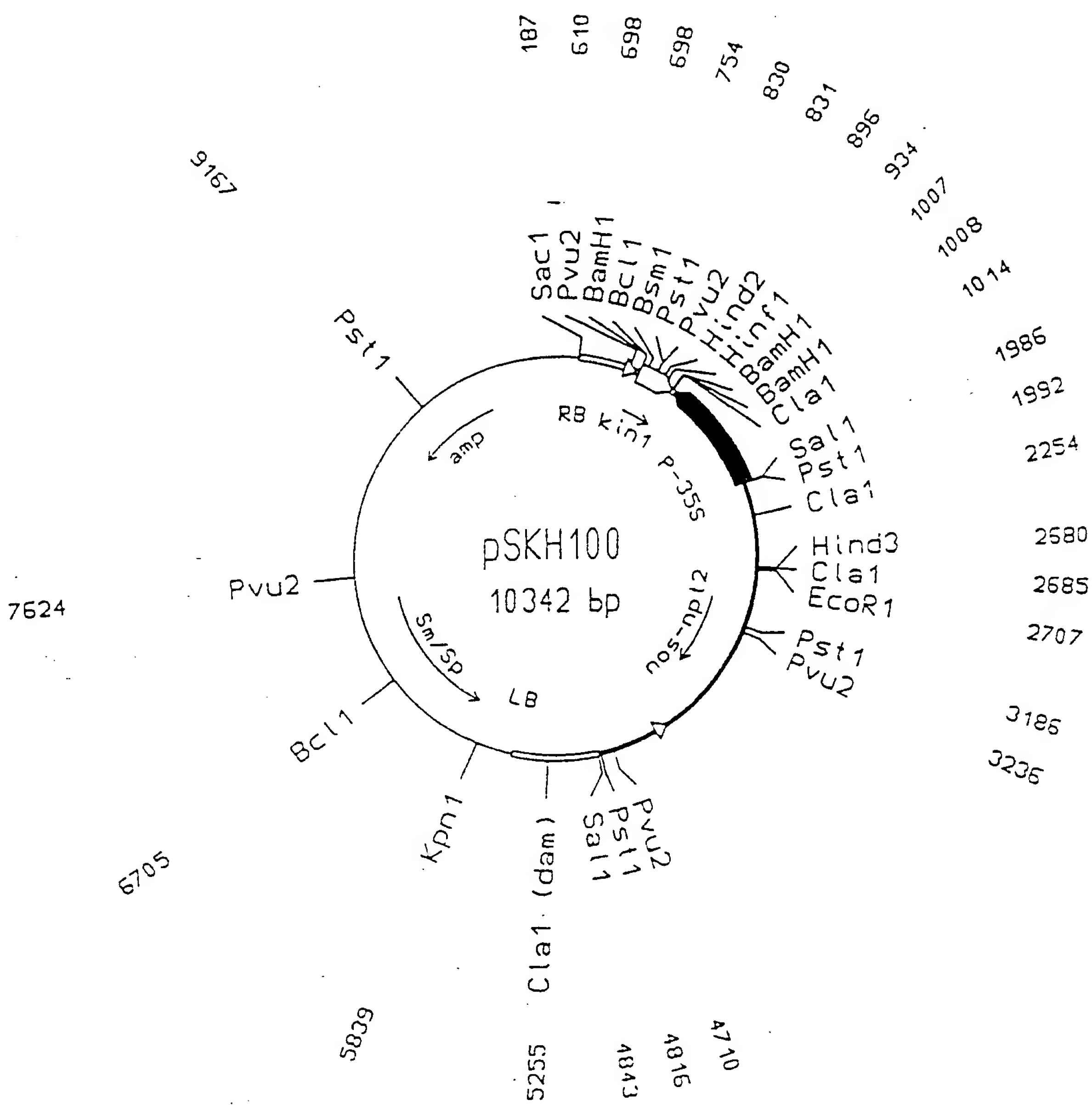

187
610
698
698
754
830
831
896
934
1007
1008
1014
1986
1992
2254
2580
2685
2707
3185
3236
4710
4816
4843
5255
5839
6705
7624
9167
Sac1
Pvu2
BamH1
Bcl1
Bsm1
Pst1
Pvu2
Hind2
Hinf1
BamH1
BamH1
Cla1
Sal1
Pst1
Cla1
Hind3
Cla1
EcoR1
Pst1
Pvu2
Pvu2
Pst1
Sal1
Cla1 (dam)
Kpn1
Bcl1
Pvu2
Pst1
pSKH100
10342 bp
RB
kin1
P-35S
nos-npt2
LB
Sm/Sp
amp

FIG. 7

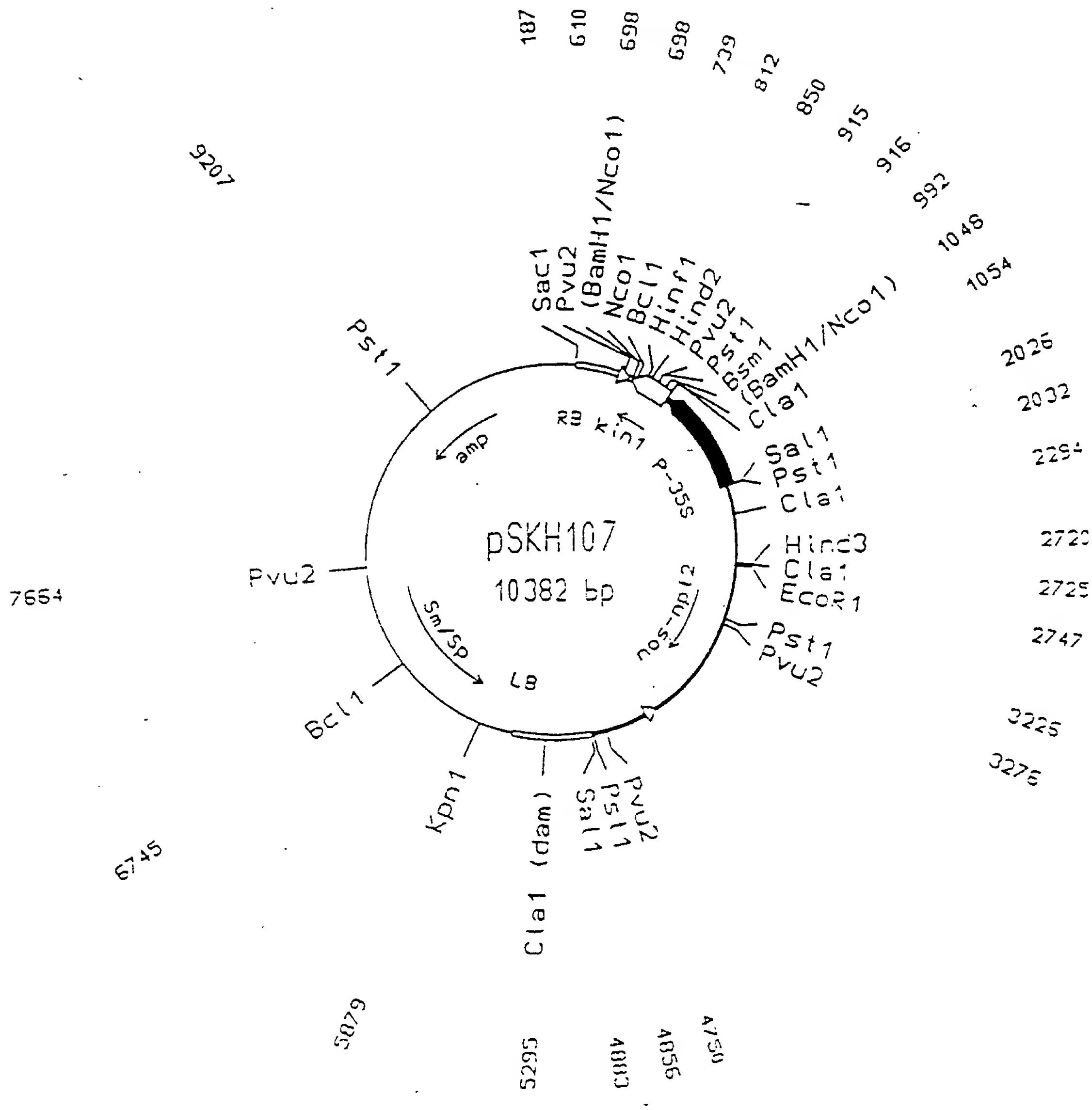
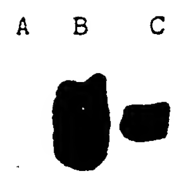
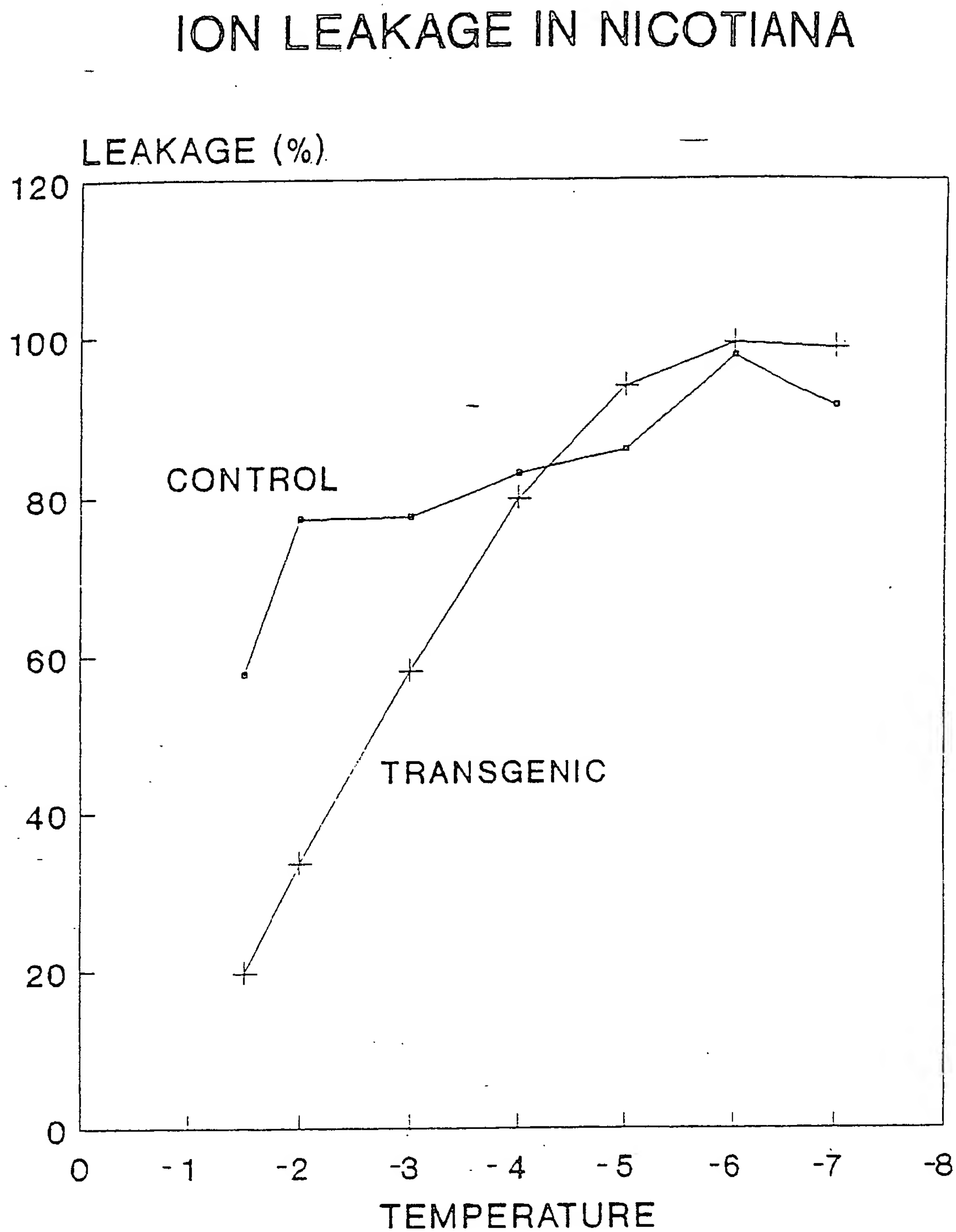

pSKH107
10382 bp
Sac1
Pvu2
(BamH1/Nco1)
Nco1
Bcl1
Hinf1
Hind2
Pvu2
Pst1
Bsm1
(BamH1/Nco1)
Cla1
Sal1
Pst1
Cla1
Hind3
Cla1
EcoR1
Pst1
Pvu2
Pvu2
Pst1
Sal1
Cla1 (dam)
Kpn1
Bcl1
Pvu2
Pst1
amp
RB
kin1
P-35S
nos-npt2
Sm/Sp
LB
187
610
698
698
739
812
850
915
916
992
1046
1054
2026
2032
2294
2720
2725
2747
3226
3276
4750
4856
4880
5295
5879
6745
7654
9207

FIG. 8



A= non-transformed tobacco
B= acclimated Arabidopsis RNA
C= tobacco transformed with p35S-sense-kin1

FIG. 9

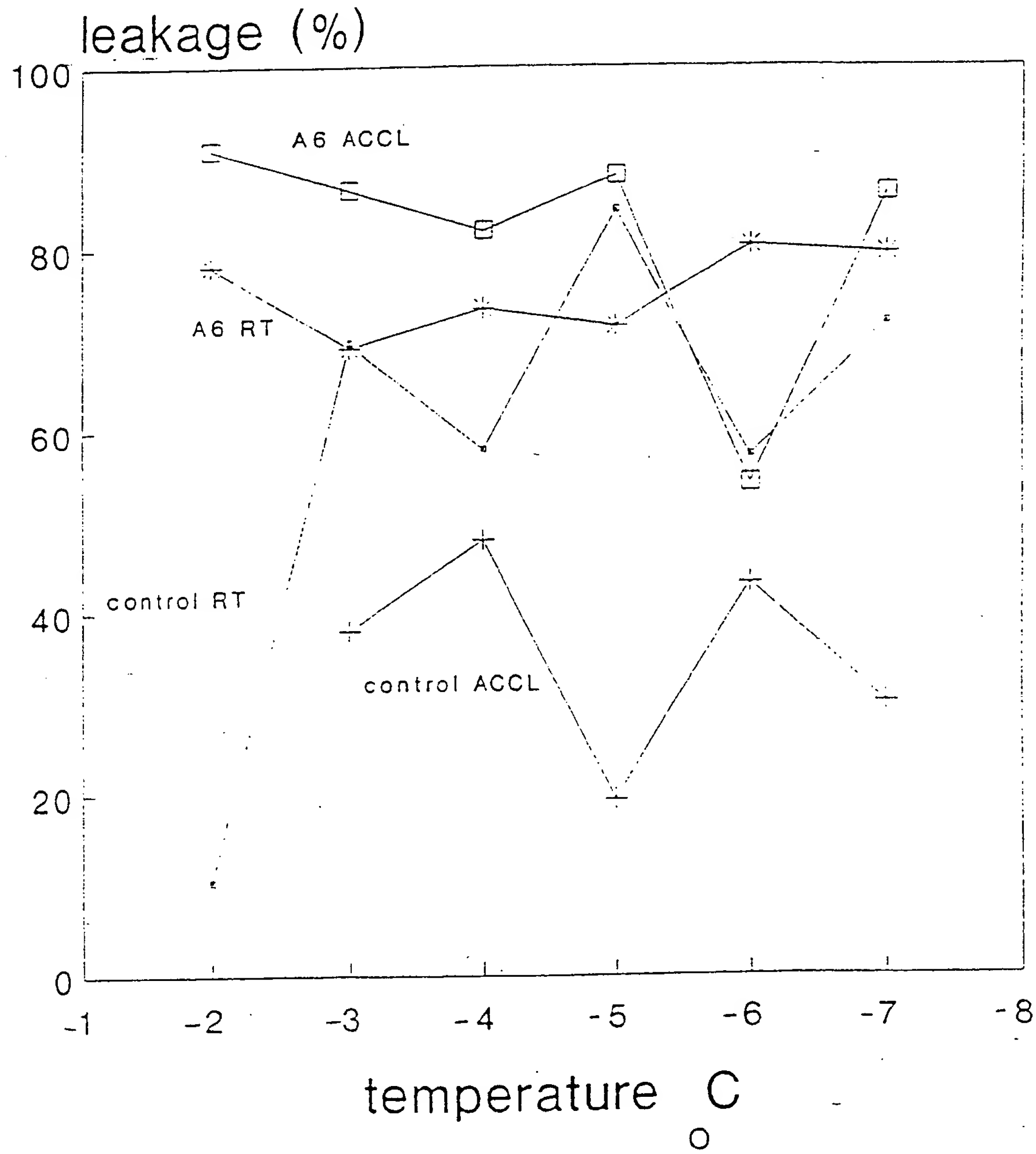


CONTROL= wild type Nicotiana
TRANSGENIC= Nicotiana transformed with
p35S-kin1

FIG. 10

ION LEAKAGE IN ARABIDOPSIS

control and antisense



RT= +20 C ACCL= acclimatized +4 C
control = non-transformed
A6=transformed with p35S-antisense-kin1

FIG. 11

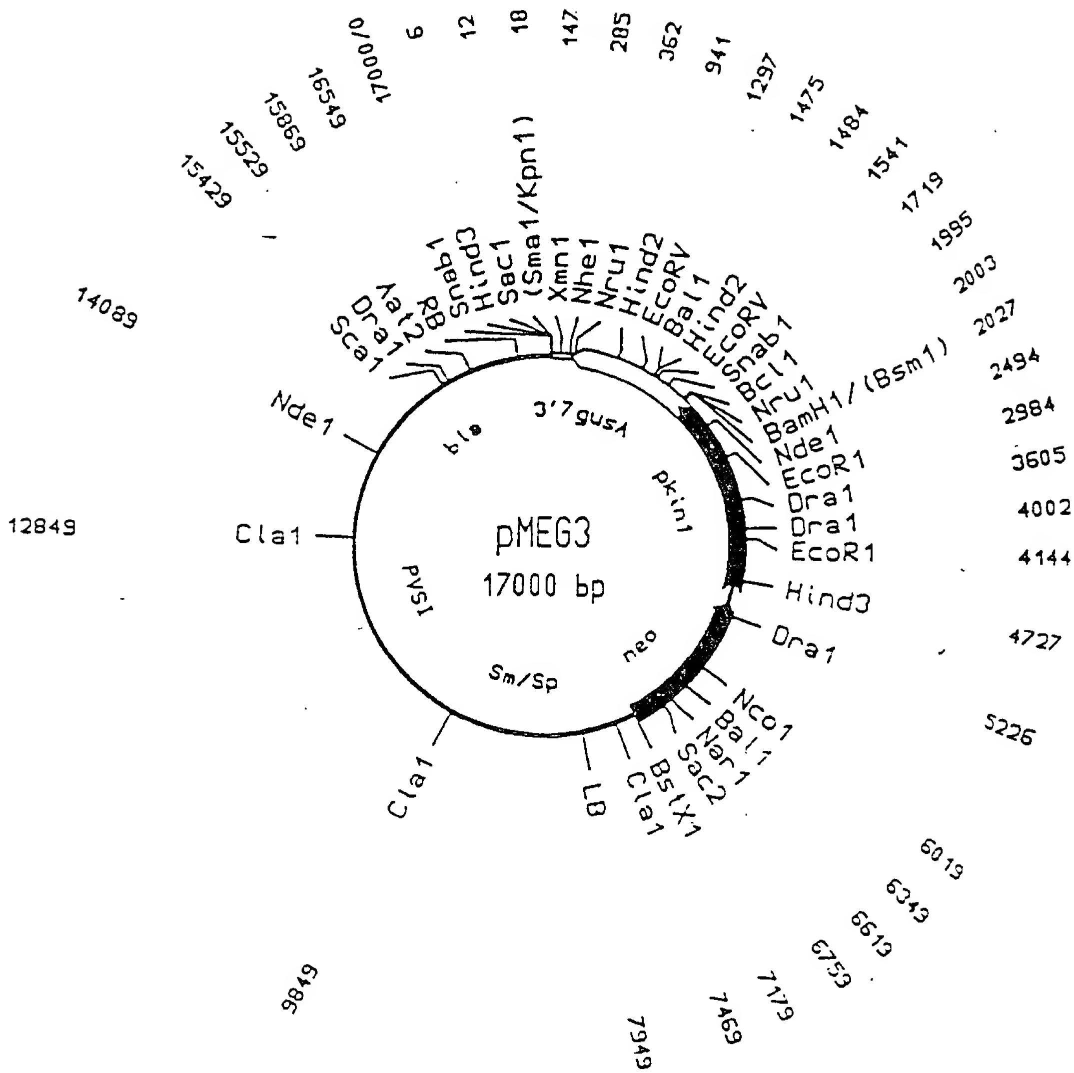

pMEG3
17000 bp
3'7gusA
bla
pkin1
pVS1
Sm/Sp
neo
Sca1
Dra1
Aat2
RB
Snab1
Cpub1
Hind3
Sac1
(Sma1/Kpn1)
Xmn1
Nhe1
Nru1
Hind2
EcoRV
Bal1
Hind2
EcoRV
Snab1
Bcl1
Nru1
BamH1/(Bsm1)
Nde1
EcoR1
Dra1
Dra1
EcoR1
Hind3
Dra1
Nco1
Bal1
Nar1
Sac2
BstX1
Cla1
LB
Cla1
Cla1
Nde1
17000/0
6
12
18
147
285
362
941
1297
1475
1484
1541
1719
1995
2003
2027
2494
2984
3605
4002
4144
4727
5226
6019
6343
6613
6753
7179
7469
7949
9849
12849
14089
15429
15529
15869
16549

FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 90/00284

I. CLAS[illegible]CATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

Accordi[illegible] [illegible]ternational Patent Classification (IPC) or to both National Classification and IPC

IPC5: [illegible] 2 N 15/29, C 12 N 15/82, A 01 H 4/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 N; A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	EP, A2, 0338266 (THE GENERAL HOSPITAL CORPORATION) 25 October 1989, see the whole document --	1-11
P,X	Plant Molecular Biology, Vol. 15, 1990 Sirpa Kurkela et al.: "Cloning and characterization of a cold- and ABA-inducible Arabidopsis gene", see page 137 - page 144 the whole article --	1-11
X	Plant Physiol, Vol. 87, 1988 Sarah J. Gilmour et al.: "Cold Acclimation in Arabidopsis thaliana", see page 745 - page 750 whole article --	1-3,10-11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd February 1991	1991 -02- 27
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Supplement to Plant Physiology, Vol. 89, No. 4, 1989 Gilmour et al.: "Scientific program for the Annual Meeting of the American Society of Plant Physiologists", abstract no. 802 --	1-3,10-11
X	Hajela et al."Journal of Cellular Biochemistry, Supplement 13 D", 1989, Alan R. Liss, Inc.,, abstract no. 417 --	1-3,10-11
X	J. Plant Physiol., Vol. 135, 1989 Adrian J. Cutler et al.: "Winter Flounder Antifreeze Protein Improves the Cold Hardiness of Plant Tissues", see page 351 - page 354 the whole article --	1,10-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/FI 90/00284

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-01-31
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0338266	89-10-25	AU-D- 3346489	89-10-16
		WO-A- 89/09219	89-10-05